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PROCESS FOR THE PREPARATION OF A HYDROLYZED SQUASH OIL HYDROLYZED SQUASH OIL CAPABLE OF BEING OBTAINED BY THIS PROCESS AND ITS UTILIZATION

[Procede de Preparation d'une Huile de Courge hydrolysee, l'Huile de Courge hydrolysee susceptible d'etre Obtenue par ce Procede et son Utilisation]

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/1[1]

This invention relates to a process for the preparation of a hydrolyzed squash oil, the hydrolyzed squash oil capable of being obtained by that process, its pharmaceutical and cosmetic use or as food supplement and compositions containing it.

Squash by the Latin name of *Cucurbita pepo* belongs to the family of Cucurbitaceae. This is a big annual herbaceous plant with an angular stalk with very many branches and spreading on the ground. The leaves are simple, alternating and longitudinally petiolulate. The flowers are yellow and unisex. The fruit is a big, voluminous or oblong berry with a meaty pulp, and it is spongy. Its color varies. There are numerous flat ovoid seeds with a length of 7 to 15 mm and a thickness of 8 to 9 mm.

The plant flowers from July to August. The plant is cultivated and reportedly originated in Central America.

The kernels on the average consist chemically of 35 to 50% oil, 25 to 40% proteins, 10% pectins and 4 to 5% mineral elements. The oil is the most worthwhile part and is currently used.

In what follows, we will use both the terms "squash oil" and "squash kernel oil."

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[1] Numbers in the margin indicate pagination in the foreign text.

This is a highly unsaturated oil whose composition in terms of fatty acid is as follows:

- palmitic acid C16:0 from 10 to 15%
- stearic acid C18:0 from 4 to 10%
- oleic acid C18:1 from 30 to 40%
- linoleic acid C18:2 from 40 to 50%.

The fatty acids are mostly tied to glycerol to form /2 triglycerides. Squash oil on the average contains between 1.5 and 2.5% unsaponifiable substance. The unsaponifiable fraction of a fatty body or the "unsaponifiable" is defined by the assembly of constituents, which, after the saponification of the oil, are soluble in the conventional solvents used for fatty bodies (hexane, ethyl oxide, etc.). The unsaponifiable is a complex mixture of compounds, some of which are present in the state of traces and which belong to widely different families. Thus, the unsaponifiable portion of squash oil is made up of hydrocarbons, tocopherols (average contents 650 mg/kg, while the γ-tocopherol is the majority compound), triterpenic alcohols (average content 2 g/kg), methyl-4-sterols and sterols. The latter are made up mostly of delta-7-sterols at a level of 95% and 5% of delta-5-sterol. Present among these sterols are campesterol, stigmasterol, sitosterol, spinosterol, stigmastotriene-7,22,25-ol, stigmastodiene-7,25-ol, stigmastene-7-ol and stigmastodiene-7,24-ol. The rich content of delta-7-

sterol is a chemical characteristic of the unsaponifiable portion of squash oil.

Squash oil is conventionally and essentially used *per os* as a complement in treating prostatic functional disorders and diuresis difficulties. It is said that squash oil works as a prostatic decongestant. Among prostate function disorders, one can especially mention the benign hypertrophy of the prostate or prostatic adenoma. This hypertrophy is an affliction that strikes many men from the age of 40 to 50 onward and that is manifested by the proliferation of prostatic tissue. At the age of 60, 50% of men are stricken, and at age 85, 90% are so stricken. But many of them remain asymptomatic throughout their lives.

Few plants are used in phytotherapy to reduce the functional symptomatology of the benign hypertrophy of the prostate. These plants are *Serenoa repens, Urtica dioica, Pygeum africanum* and *Cucurbita pepo*. The inhibition of the 5α-reductase enzyme is the manner of action that is tied to the prostatic decongesting effect of the squash oil *Cucurbita pepo*. The molecules that are behind this activity are the delta-7-sterols contained in the unsaponifiable portion. Thus, a clinical study done on a mixture of its compounds confirms this activity (Bach D. Schmitth, Ebeling GL (1996) /3

Phytopharmaceutical and Synthetic Agents in the Treatment of Benign Prostatic Hyperplasia - Phytomedicine 3 (4), 309-313). The authors explain the activity observed here by the conformational analogy of the delta-7-serols with dihydrosterone, which one does not encounter in the delta-5-sterols.

Squash oil is furthermore used for the treatment of fatty skin. It is known that one can treat fatty skins by a 5α-reductase inhibitor, which is an enzyme in hyperseborrhea. This latter pathology can especially cause hair loss and androgenogenetic alopecia.

5α-reductase is an enzyme that was discovered, above all, in the prostate. It catalyzes the conversion of testosterone into dihydrotestosterone, which, in turn, is tied to the androgynous receptor and initiates the development of the genital parts and the prostate. More recently, 5α-reductase was found in the scalp and furthermore in the skin where the same reaction takes place as in the prostate. It is believed that disorders in the activity of 5α-reductase can cause alopecia, acne and hirsutism.

It has now been found that the activity that inhibits 5α-reductase of squash oil could be boosted when it undergoes a stage of hydrolysis. Thus, in this operation, the triglycerides in part or totally release fatty acids in a free form.

The fatty acids in effect are tied to glycerol by ester bonds. In the context of this invention, these bonds can then be hydrolyzed by chemical means (saponification) or by enzymatic means with an esterase that is also called lipase. In addition to the fact that it is natural, the enzymatic group offers the advantage of being more specific and causes less degradation. It will thus be preferred in the context of this invention.

The object of this invention thus is a process for preparing a hydrolyzed squash oil, characterized in that it contains a hydrolyzed phase of oil extracted from the kernels of the *Cucurbita pepo* squash.

A second object of the invention is the oil of hydrolyzed squash capable of being obtained by the process according to the invention.

Finally, the invention also relates to this hydrolyzed /4 squash oil for application as a medication and its use for the preparation of a medication intended to fight against all the pathologies or all the disorders that come under the heading of dysfunction of 5α-reductase activity.

Hydrolyzed squash oil can be administered orally or topically.

It can also be used for the preparation of a medication intended to treat prostate function disorders among which we might mention prostate hypertrophy and prostate adenoma,

alopecia, hyperseborrhea, hirsutism and acne. A pharmaceutical composition for administration orally containing a hydrolyzed squash oil according to this invention and a dermatological composition for topical administration containing said same hydrolyzed squash oil are also a part of the invention.

The invention furthermore relates to a cosmetic composition for topical application containing hydrolyzed squash oil, which is the object of this invention. Such a cosmetic composition can make it possible to improve the appearance of the skin.

Finally, a food composition or food complement containing hydrolyzed squash oil as an object of this invention is also a part of the invention.

A pharmaceutical composition for oral administration can comprise 10 mg to 2 g of hydrolyzed squash oil according to the invention and preferably between 1 and 100% by weight with relation to the total weight of the composition. According to the invention, the pharmaceutical composition, as a matter of fact, can come in the form of a soft capsule on a base of glycerin or gelatin containing pure hydrolyzed squash oil without any additive.

A dermatological composition for topical administration can comprise between 0.01% to 30% by weight of hydrolyzed squash oil according to the invention, preferably between 0.3 and 5% by weight with relation to the total weight of the composition.

A cosmetic composition can comprise between 0.01 and 30% by weight of hydrolyzed squash oil according to the invention, preferably between 0.3 and 5% by weight with respect to the total weight of the composition.

A food composition or food complement may comprise /5 between 1 and 100% by weight of hydrolyzed squash oil according to the invention. The daily dose of hydrolyzed squash oil can thus be ingested in the form of a food composition and can comprise between 10 mg and 10 g.

The galenic formulations of the pharmaceutical and dermatological compositions can be made according to methods known to experts in the field.

According to a particular aspect of the invention, squash oil is advantageously emulsified while stirring in an aqueous solution prior to the hydrolysis phase. This aqueous solution is preferably buffered at a pH varying between 7 and 9 and the temperature advantageously is varied between 25 and 45°C. The aqueous solution, in particular, can be buffered with the help of sodium hydrogenophosphate (Na_2HPO_4).

Among the microorganisms that produce lipases that are suitable for the process according to the invention, we might mention *Thermomyces lanuginosus*, also called *Humicola lanuginosa*, *Aspergillus oryzae*, *Rhizomucor miehei*. Novozym®

27007 and Novozym® 398, sold by the NOVO Company, are particularly suitable in connection with lipase.

The ratio between squash oil and aqueous solution can vary from 4/1 to 1/4.

In the case where the hydrolysis is done by a lipase, the process can be described in the following fashion:

- To the previously prepared emulsion, we add a lipase in a proportion with relation to the emulsion thus created that will vary from 1/0.1 to 1/0.001.
- We maintain the temperature and keep stirring throughout the entire hydrolysis.
- We keep the pH at 6 throughout the entire operation of hydrolysis by means of the successive addition of a buffer solution.

The enzymatic solution can be followed by the determination of the acid index of the oily part. The enzymatic hydrolysis can be total within a period varying between 1 and 10 hours.

Optionally, one can proceed to a liquid/liquid /6 separation of the oily and aqueous phases. This operation can be done by simple decantation, by centrifugation or by adding an organic solvent such as acetone, an alcohol of C_1 to C_4, hexane or dichloromethane. In this case, one evaporates the solvent after separation. If one does not wish to separate the two liquid, aqueous and oily phases, one can stabilize the emulsion

by means of a conventional emulsifier. Thus, the emulsion can be used directly in the pharmaceutical applications described below, especially orally or topically.

The acid index of hydrolyzed squash oil can be determined by the technique of the French Pharmacopoeia. It varies from 100 to 200 once the hydrolysis has been done. The fatty acid section can be determined by CPG [gas liquid chromatography] on the hydrolyzed and methylated squash oil. The average contents greatly resemble the fatty acid section of the starting oil. Thus, the average contents can vary according to the various lots, in other words, 10 to 15% for palmitic acid, 4 to 10% for stearic acid, 30 to 40% for oleic acid, and 40 to 50% for linoleic acid. Finally, we determined the content of unsaponifiable substance of the hydrolyzed squash oil: It is the same as that of the starting oil, in other words, on the average, it is between 1.5 and 2.5%.

The examples below will illustrate this invention without, however, confining its scope.

Example 1

100 kg of squash oil are inserted in a thermostat-controlled reactor while stirring. We add 65 kg of an aqueous solution of 20% Na_2HPO_4 with a pH of 8.5. When the temperature of the emulsion attains 37°C, we add 1 kg of a Novozym® 27007 lipase. We keep stirring and we maintain the pH at 6 by adding

initial buffer solution and by maintaining the temperature throughout the entire hydrolysis. We regularly sample an aliquot part of the emulsion with which one performs the determination of the acid index. When the latter attains a value of 140, we add 100 kg of acetone to the emulsion and we perform liquid/liquid extraction. The organic phase is isolated; the acetone is evaporated in a vacuum. We thus recover hydrolyzed squash oil.

Its acid index is confirmed at 140 along with the /7 similarity of the fatty acid section with the initial oil and its unsaponifiable portion.

Example 2

1 kg of squash oil is inserted in a reactor while stirring. The temperature is regulated at 40°C. We add 3 kg of a buffered aqueous solution of 20% Na_2HPO_4 with a pH of 8. When the temperature of the emulsion attains 40°C, we add 200 g of a Novozym® 398 lipase. The pH of the emulsion is kept at 6 by adding initial buffer solution The stirring and the temperature remain constant throughout the entire hydrolysis. The latter is followed by the determination of the acid index. We stop the stirring when the latter attains a constant value of 150. The emulsion is centrifuged so as to separate the hydrolyzed oil from the aqueous phase. The hydrolyzed squash oil obtained has an acid index close to 150; its fatty acid portion corresponds

to that of the initial oil as well as its unsaponifiable portion.

Example 3

Test on the production of 5α-reductase

The chemical characterizations of hydrolyzed squash oil show that they contain the unsaponifiable part of the squash oil associated with fatty acids in the free form.

We test the activity with regard to the inhibition of the production of 5α-reductase on fibroblasts of hydrolyzed squash oil, the conventional squash oil as well as a reconstituted oil without unsaponifiable parts but made up of the association of majority fatty acids ≅ 13% palmitic acid, 6% stearic acid, 35% oleic acid and 45% linoleic acid.

The test system consists of normal human dermic /8 fibroblasts used in a single layer. The fibroblasts are isolated from a plastic surgery. The fibroblast incubation environment consists of a nutritive environment to which we have added a tritiated testosterone and marked non-radio [non-x-ray] testosterone. The fibroblasts are pre-incubated in the presence of the product to be tested for a period of 24 hours. After this period of time, we subject the cells to bacterial lysis. The lysates obtained are extracted with dichloromethane. After evaporation, the dry residues are picked up in methanol and are placed on silica plates and then chromatographed.

At the end of the migration, the radio activity of the separated spots is counted by a radio activity analyzer. The metabolization of the testosterone into 5-α-dihydrotestosterone by 5α-reductase is calculated. They are expressed in terms of percentages of the activity of 5α-reductase present in the control.

Inhibition of 5α-reductase activity	
Squash oil	
0.1 mg/ml	No activity
0.02 mg/ml	No activity
Reconstituted squash oil	
0.1 mg/ml	- 18%
0.02 mg/ml	- 7%
Hydrolyzed squash oil	
0.1 mg/ml	- 45%
0.02 mg/ml	- 27%

In this test, the squash oil containing the /9
Unsaponifiable portion does not display any activity at the concentrations tested, whereas the reconstituted squash oil, made up of free fatty acids without unsaponifiable parts, displays an activity which, however, is less than that of hydrolyzed squash oil containing the unsaponifiable parts at the same concentration. We thus note a synergy of action between the unsaponifiable parts and the fatty acids in the free form, which explains the superiority of the action of hydrolyzed squash oil on the inhibition of 5α-reductase.

Example 4

Inhibiting activity of 5α-reductase on the costo-vertebral gland of the hamster

This test involves treatment with the help of the control product or testing of male hamsters that were castrated by massage of the costo-vertebral glands for a period of 4 weeks. The parameters that are tracked are the surface and the histological study of the glands.

The untreated castrated animals show a major diminution of the size of the glands at 29 days.

The animals treated by testosterone show a noticeable increase in the surface of the glands, +34% at 29 days.

The products to be tested are applied in association with testosterone. The 5α-reductase-inhibiting activity is expressed by a nonmetabolization of the inactive testosterone into active dihydrotestosterone and thus by an inhibition of the increase in the surface of the costo-vertebral glands of the hamsters.

The hydrolyzed squash oil is applied diluted to 60% and 30% in a neutral solvent. Next, 3% flutamide is used as active control. Flutamide is an antagonist of receptors for androgens, making it possible to check on the growth of the dependent androgynous cancer cells.

As regards the parameter represented by the surface of the costo-vertebral glands, hydrolyzed squash oil, diluted to 60% over a certain period of time, shows a significant reduction.

On the other hand, 30% diluted hydrolyzed squash oil does not display any activity.

The histological study of the glands makes it possible /10 to determine the maximum diameter of each globular mass and the thickness of the interglandular epidermis. Other parameters can be studied, but in the test performed, even the positive control, in other words, flutamide, will not display any active behavior. These parameters thus cannot be used.

As for the maximum parameter of the costo-vertebral gland, flutamide, a control product, reduces the action of the testosterone with regard to the increase in the maximum diameter by 84.2%. Furthermore, 30% diluted hydrolyzed squash oil reduces it by 39.12% and 60% diluted hydrolyzed squash oil reduces it by 53.9%.

Treatment with testosterone permits normal histological development of the thickness of the epidermis of the gland. On the other hand, animals treated with flutamide show a 28% increase in the thickness of their epidermis. Besides, 30% diluted hydrolyzed squash oil does not have any repercussions; hydrolyzed squash oil, on the other hand, causes a 29% increase in the thickness.

Example 5

Foaming gel without soap

Ingredients	Quantity (g)

Lauryl ether sulfate 70%	0-12
Cocamidopropylbetaine	0-20
Decyl glucoside	0-15
Polysorbate 20	0-10
Sodium lauryl sarcosinate	0-15
Ceteareth-60 myristyl glycol	0-10
Glycerin	0-10
Methyl gluceth-20	0-5
Zinc gluconate	0-1
Cetrimonium chloride	0-0.5
Oil of hydrolyzed Cucurbita pepo	0.01-5
Conservation agents	qs
Perfume	qs
Coloring agent	qs
Demineralized water	qsp 100 g

Example 6 /11

H/E emulsion

Ingredients	Quantity (g)
Sepigel 305	2-10
Cyclomethicone	0-10
Propylene glycol	0-10
Glycerin	0-7
Polymethyl methacrylate	0-3
Zinc gluconate	0-1

Cetrimonium chloride	0-0.5
Salicylic acid	0-1
Oil of hydrolyzed Cucurbita pepo	0.01-5
Triethanolamine	qs pH
Perfume	qs
Conservation agents	qs
Demineralized water	qsp 100 g

CLAIMS /12

1. Process for the preparation of a hydrolyzed squash oil, characterized in that it contains a hydrolysis phase of the extracted oil of kernels of the squash *Cucurbita pepo*.
2. Process for the preparation of a hydrolyzed squash oil according to Claim 1, characterized in that the hydrolysis is performed with the help of a lipase.
3. Process for the preparation of a hydrolyzed squash oil according to Claim 2, characterized in that the microorganisms that produce lipase are chosen from among *Thermomyces lanuginosus*, also called *Humicola lanuginosa*, *Aspergillus oryzae* and *Rhizomucor miehei*.
4. Process for the preparation of a hydrolyzed squash oil according to any of Claims 1 or 3, characterized in that the oil extracted from kernels of squash is, prior to the hydrolysis phase, emulsified while being stirred in an aqueous solution.

5. Process for the preparation of a hydrolyzed squash oil according to Claim 4, characterized in that the aqueous solution is buffered at a pH varying between 7 and 9 and is characterized in that the emulsion is kept at a temperature of between 25 and 45°C.

6. Process according to any of Claims 4 or 5, characterized in that the ratio between extracted oil of squash kernels and aqueous solution varies between 4/1 and 1/4.

7. Process according to any of Claims 4 to 6, characterized in that the hydrolysis phase by way of a lipase is followed by a liquid/liquid separation of the oil and aqueous phases.

8. Process according to Claim 7, characterized in that the separation of the liquid/liquid phases from the oily and aqueous phases is performed by means of decantation, by centrifugation or by adding an organic solvent.

9. Process according to Claim 8, characterized in that the organic solvent is selected from among acetone, C_1 to C_4 alcohol, hexane or dichloromethane.

10. Process according to any of Claims 4 to 9, characterized in that one adds an emulsifier to stabilize the emulsion thus obtained.

11. Hydrolyzed squash oil capable of being obtained by the /13 process according to any of Claims 1 to 10.

12. Hydrolyzed squash oil according to Claim 11, characterized in that the acid index is between 100 and 200.

13. Hydrolyzed squash oil according to Claim 11 and 12, characterized in that it contains 4 to 10% stearic acid, 30 to 40% oleic acid, and 40 to 50% linoleic acid with relation to the total weight of the hydrolyzed squash oil.

14. Hydrolyzed squash oil according to Claim 13, characterized in that its content of unsaponifiable substance is between 1.5 and 2.5% with respect to the total weight of the hydrolyzed squash oil.

15. Hydrolyzed squash oil according to any of Claims 11 to 14 for use as medication.

16. Hydrolyzed squash oil according to Claim 15 for use as inhibitor of 5α-reductase.

17. Use of a hydrolyzed squash oil according to any of Claims 11 to 14 for the preparation of a medication to fight all pathologies or all disorders having to do with a dysfunction of the activity of 5α-reductase.

18. Use of a hydrolyzed squash oil according to Claim 17, characterized in that the medication can be administered orally or topically.

19. Use of a hydrolyzed squash oil according to any of Claims 11 to 14 for the preparation of a medication intended for the treatment of prostate function disorders.

20. Use of a hydrolyzed squash oil according to any of Claims 11 to 14 for the preparation of a medication intended for the treatment of alopecia or hyperseborrhea, hirsutism or acne.

21. Cosmetic use of hydrolyzed squash oil according to any of Claims 11 to 14 to improve the appearance of the skin.

22. Cosmetic use of hydrolyzed squash oil according to any of Claims 11 to 14 as a food composition or supplement.

23. Pharmaceutical composition for administration via the /14 oral route containing a hydrolyzed squash oil according to any of Claims 11 to 14.

24. Dermatological composition for optical administration containing a hydrolyzed squash oil according to any of Claims 11 to 14.

25. Cosmetic composition for administration via the topical route containing a hydrolyzed squash oil according to any of Claims 11 to 14.

26. Food composition or food supplement for administration via the oral route containing a hydrolyzed squash oil according to any of Claims 11 to 14.